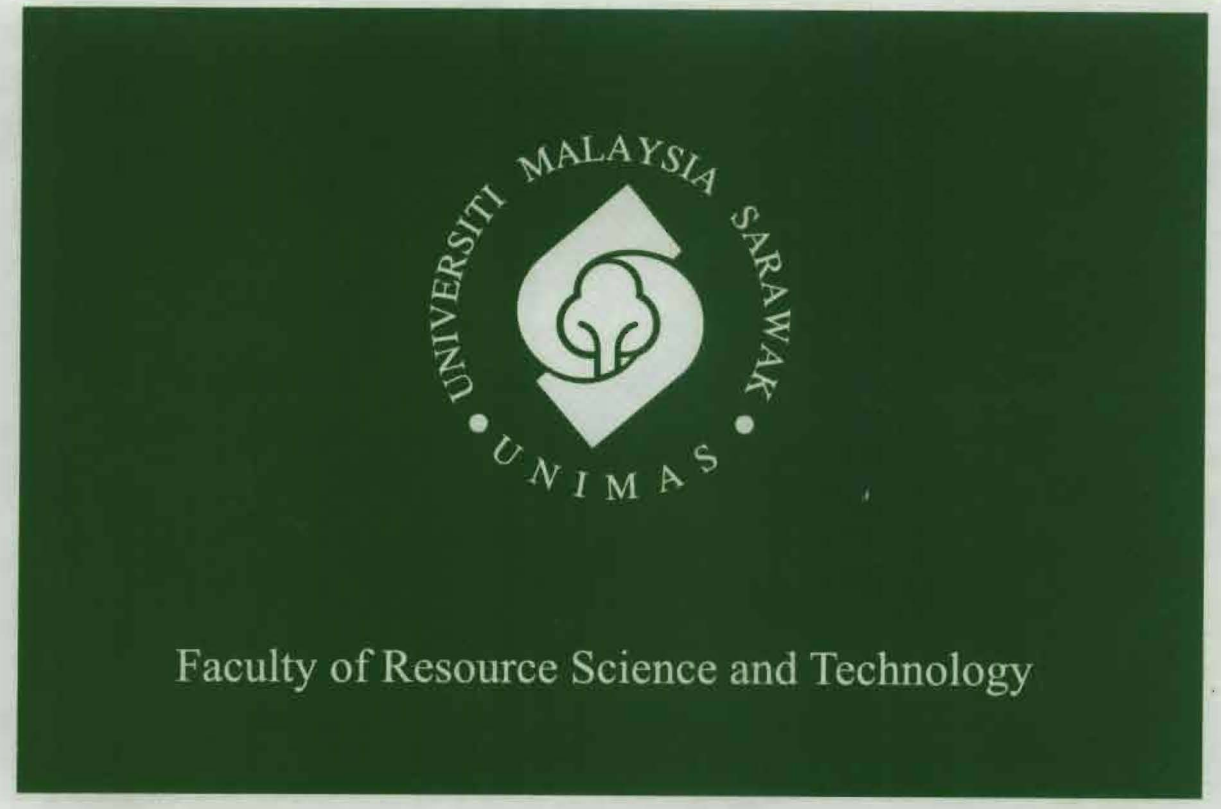


ISOLATION AND CHARACTERIZATION OF INDIGENOUS PLANT GROWTH PROMOTING ENDOPHYTIC BACTERIA FROM SAGO PALM ROOT

Anysia Hedy binti Ujat

(40610)

Bachelor of Science with Honours
(Resource Biotechnology)
2016

QK
495
P17
A637
2016

UNIVERSITI MALAYSIA SARAWAK

Grade: _____

Please tick (√)
Final Year Project Report ☑
Masters ☐
PhD ☐

DECLARATION OF ORIGINAL WORK

This declaration is made on the ....30.....day of...JUNE.....2016.

Student's Declaration:

I ANYSIA HEDY BINTI UJAT, 40610, FACULTY OF RESOURCE SCIENCE AND TECHNOLOGY
- (PLEASE INDICATE STUDENT'S NAME, MATRIC NO. AND FACULTY) hereby declare that the work entitled ISOLATION AND CHARACTERIZATION OF INDIGENOUS PLANT GROWTH PROMOTING ENDOPHYTIC BACTERIA FROM SAGO PALM ROOT is my original work. I have not copied from any other students' work or from any other sources except where due reference or acknowledgement is made explicitly in the text, nor has any part been written for me by another person.

30 June 2016
Date submitted

ANYSIA HEDY BINTI UJAT (40610)
Name of the student (Matric No.)

Supervisor's Declaration:

I AP DR AWG AHMAD SALLEHIN AWG HUSAINI (SUPERVISOR'S NAME) hereby certifies that the work entitled ISOLATION AND CHARACTERIZATION OF INDIGENOUS PLANT GROWTH PROMOTING ENDOPHYTIC BACTERIA FROM SAGO PALM ROOT (TITLE) was prepared by the above named student, and was submitted to the "FACULTY" as a * partial/~~full~~ fulfillment for the conferment of BACHELOR OF SCIENCE WITH HONORS (RESOURCE BIOTECHNOLOGY) (PLEASE INDICATE THE DEGREE), and the aforementioned work, to the best of my knowledge, is the said student's work.

Received for examination by: _____ Date: 30 June 2016

(Name of the supervisor)
Assoc Prof Dr Awg Ahmad Sallehin Awg Husaini
Deputy Director II
Research and Innovation Management Centre
Universiti Malaysia Sarawak

I declare that Project/Thesis is classified as (Please tick (√)):

☐ **CONFIDENTIAL** (Contains confidential information under the Official Secret Act 1972)*
☐ **RESTRICTED** (Contains restricted information as specified by the organisation where research was done)*
☑ **OPEN ACCESS**

Validation of Project/Thesis

I therefore duly affirm with free consent and willingly declare that this said Project/Thesis shall be placed officially in the Centre for Academic Information Services with the abiding interest and rights as follows:

- This Project/Thesis is the sole legal property of Universiti Malaysia Sarawak (UNIMAS).
- The Centre for Academic Information Services has the lawful right to make copies for the purpose of academic and research only and not for other purpose.
- The Centre for Academic Information Services has the lawful right to digitalise the content for the Local Content Database.
- The Centre for Academic Information Services has the lawful right to make copies of the Project/Thesis for academic exchange between Higher Learning Institute.
- No dispute or any claim shall arise from the student itself neither third party on this Project/Thesis once it becomes the sole property of UNIMAS.
- This Project/Thesis or any material, data and information related to it shall not be distributed, published or disclosed to any party by the student except with UNIMAS permission.

Student signature _____ (Date)

Supervisor signature: _____ (Date)

Current Address:
587 TAMAN BANDAR JAYA, JALAN TUN HUSSEIN ONN, 97000 BINTULU, SARAWAK

Notes: * If the Project/Thesis is **CONFIDENTIAL** or **RESTRICTED**, please attach together as annexure a letter from the organisation with the period and reasons of confidentiality and restriction.

[The instrument is duly prepared by The Centre for Academic Information Services]

ACKNOWLEDGEMENT

My greatest appreciation and thankfulness to my supervisor, Assoc. Prof. Dr. Awang Ahmad Sallehin and also to my co-supervisor, Dr. Samuel Lihan for their guidance, assistance, and support throughout the completion of my final year project.

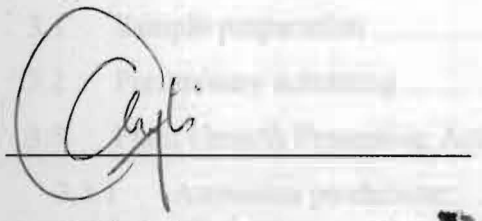
I am hugely indebted to all Molecular Genetic Laboratories postgraduate student and Molecular Biology Department lecturers and staff, for their assistance, advices and encouragement the process of completing this project. Not to forget my labmates, Mr. Safwan bin Mazlan, Ms Nurfarhanah bt Roslan, Ms Syahirah Nadiah bt Mohd Johari, Ms Izzatul Syazana bt Ismail and Ms Syifa' bt Jamaludin that constantly discussing and exchanging ideas and opinion with me.

Special thanks to my parents, Mr Ujat bin Kadir and Mdm Teo Swee Aie and also my siblings who continuously give me moral support and encouragement in my life.

Lastly I would like to thank all my friends those who are always there to lend me a helping hand and being supportive to me upon finishing this project.

DECLARATION

I hereby declare that this thesis is based on my original work except for quotation and citation, which have been duty acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UNIMAS or other institutions.



Anysia Hedy binti Ujat

Resource Biotechnology

Department of Molecular Biology

Faculty of Resource Science and Technology

University Malaysia Sarawak

TABLE OF CONTENT

LIST OF ABBREVIATIONS

°C	degree celcius
µL	microliter
BLAST	Basic Local Alignment Search Tool
CD	colony diameter
DNA	Deoxyribose Nucleic Acid
HCN	hydrogen cynide
HD	halo diameter
IAA	Indole-3-Acetic Acid
$MgCl_2$	magnesium chloride
NA	nutrient agar
NB	nutrient broth
NBCI	National Center for Biotechnology Information
NH_3	ammonia
PBM	phosphate solubilizing microbes
PCR	Polymerase Chain Reaction
PGP	Plant Growth Promoting
PGPR	Plant Growth Promoting Rhizobacteria
pH	Potential Hydrogen
RNA	Ribose Nucleic Acid
UV	ultra violet
TAE	Tris-Acetic Acid- Ethylenediaminetetraacetic acid
TE	Tris-Ethylenediaminetetraacetic acid

LIST OF FIGURE

LIST OF TABLES

Isolation and Characterization of Indigenous Plant Growth Promoting Endophytic Bacteria from Sago Palm Root

Anysia Hedy binti Ujat

(40610)

Resources Biotechnology
Department of Molecular Biology
Faculty of Resource Science and Technology
Universiti Malaysia Sarawak

ABSTRACT

Ten isolates of possible plant growth promoting endophytic bacteria had been successfully isolated form sago palm (*Metroxylon sagu* Rottb.) root. Endophytic bacteria designated as EB3.2, EB4.3, EB3.4, EB4.5 and EB4.6 were chosen for molecular identification based on their plant growth promoting traits such as phosphate solubilisation, siderophore production, indole-3-acetic acid production and ammonification. Molecular identification result shows that EB3.2, EB3.4, EB4.5 and EB4.6 are *Burkholderia tropica* while EB4.3 is *Enterobacter asburiae.* The bacterial isolates plant growth promoting trait were tested on green gram germination and the isolates shows an increase in seedling vigor index. The positive result obtained for plant growth promoting activities shows the potential of these bacterial isolates as potential candidates for the development of biofertilizer.

Keywords: Plant growth promoting, endophytic bacteria

ABSTRAK

Sebanyak sepuluh jenis bakteria endofit yang mempunyai ciri-ciri penggalak tumbuhan telah berjaya disaring dan dipencilkan dari dalam akar pokok sagu (Metroxylon sagu Rottb.). Bakteria endofit yang dilabelkan sebagai EB3.2, EB4.3, EB3.4, EB4.5 dan EB4.6 telah dipilih untuk pengenalpastian secara biologi molekul berdasarkan kepada ciri-ciri penggalak tumbuhan seperti pelarutan fosfat, penghasilan siderofor, penghasilan asid indola dan pengammoniaan. Keputusan pengenalpastian biologi molekul menunjukkan bahawa EB3.2, EB3.4, EB4.5 dan EB4.6 adalah Burkholderia tropica *manakala EB4.3 adalah* Enterobacter asburiae*. Ciri-ciri penggalak tumbuhan kesemua pencilan diuji dengan ujian percambahan menggunakan kacang hijau dan kesemua pencilan menunjukkan peningkatan indeks vigor biji benih. Keputusan positif ini menunjukkan potensi bakteria ini calon untuk diguna pakai sebagai biobaja*

Kata kunci: ciri-ciri penggalak tumbuhan, bakteria endofit

1.0 INTRODUCTION

Bacteria that colonized the root of a plant are known as rhizobacteria. Endophytic rhizobacteria reside inside the host plant and promotes the plant growth and yield from within the plant. The presences of endophytic bacteria in plants are not new discoveries as in the recent years, there are increasing studies of plant growth promoting root endophytic bacteria being carried out including sugar cane, sugar beet, maize, rice and other plants that are potential in bringing commercial benefit. There is also the successful usage of plant growth promoting bacteria as biofertilizer that is commercialized.

Chemical fertilizer is widely used in the agricultural sector to provide enough macronutrient such as nitrogen, phosphate, and potassium for the utilization of plant for growth and better yield for harvest, however, these chemical fertilizers prove to be detrimental to our environment in a long run as it could be washed away from the soil and cause pollution. Chemical fertilizer leach often occurs especially at a sloppy landscape with no riparian buffer underneath to act as a filter. This issue is critical and thus the need for new strategies to provide crops with enough nutrient that would maintain ecological balance and environmental friendly.

In the effort of reducing chemical usage to increase plant productivity to support increasing food and environment demand, researchers had been studying the positive effects of endophytic bacteria on plants, including plant growth promoting factor, antifungal mechanism, bioremediation mechanism and antimicrobial factor. Plant growth promoting bacteria are widely studied as it naturally occurs in the environmental, and has a promising potential as biofertilizer. While some of the endophytic bacteria occur naturally in plants, the possible entry points to a plant inner parts are through the plant natural opening or wounds, usually due to the elongation of the root hair. The potential of endophytic bacteria to promote growth by acting as biofertilizer and biocontrol agent is

worth to be researched as it could provide economic importance at the same time. Thus, this research is made for the following objectives:

a) To isolate endophytic bacteria presence in sago palm root (*Metroxylon sagu* Rottb.)
b) To identify and characterize the plant growth promoting endophytic bacteria isolated from sago palm root.
c) To evaluate and access the potential of the plant growth promoting endophytic bacteria isolated

2.0 LITERATURE REVIEW

2.1 Sago Palm (*Metroxylon sagu* Rottb.)

Sago palm is a species of palm in the genus Metroxylon and native to Southeast Asia. It can be found in Malaysia, Indonesia, Thailand, Brunei, Philippines, Papua New Guinea and South Pacific Region. As describe by Flach, (1997) the Metroxylon genus, only the palms of the species sagu are both hapaxanthic (once-flowering) and soboliferous (suckering). Sago palm (*Metroxylon sagu*) is of the plant that had been exploited for the starch in the trunk (Howell *et al.*, 2015). Sago palm accumulates starch in a large quantity in its trunk, ranging from 200-500kg of dry starch per palm (Yamato, 2011). Sago starch is still consumed as staple food by local people and being used as a material for noodle and cakes. Sago starch global consumption lies between 3% of the world market and its starch content is 4 times of rice, 5 times of corn and wheat and almost 17 times of tapioca as reported by Ishizaki (Bujang, 2014).

The state of Sarawak is by far the main exporter for sago starch and in 2011, 51,000 metric ton of sago starch was produced and it earned Sarawak UDS24.5 million (RM91 million) (Agricultural Statistic of Sarawak, 2011). As stated in Ecosol Report in 2005, due to the increasing demand of sago starch in food industries, Land and Custody Development Authority of Sarawak (LCDA) in 1997 establishes the first world commercial sago plantation in Mukah where 15,00 hectares of peat were cleared by PELITA Sago Plantation (Howell *et al.*, 2015).

2.2 Endophytic Bacteria

Plant-associated bacteria had been extensively studied and reviewed by researchers (Gopalakrishnan *et al.*, 2015) and endophytic bacteria are known to have beneficial effects on the host plant (Eevers *et al.*, 2015) without causing harm to the host plant (Deeb *et al* 2013). The word endophyte, if taken literally means "in the plant" (endon Gr. = within, phyton = plant) (Hallman *et al.*, 2006). Endophyte resides within the host plant without causing harm to the host and is more exposed to specific and stable habitat (Eevers, *et al.*, 2015). Eevers, *et al.*, (2015) in their paper stated that between endophyte, there are obligate endophytes described by Cross *et al.*, (2013) and facultative endophyte as described by Kamnev *et al.*, (2005). Endophytic bacteria may confer advantages to host plant by acting as a source of secondary metabolites and biocontrol agent (Paul *et al.*, 2012).

2.3 Plant Growth Promoting Endophytic Bacteria

Some endophytes are involved in plant growth promotion such as in the production of phytohormone (Hardoim *et al.*, 2015). Bacteria that resides in root tissue (endophyte) that inhibit space between cortical cell are referred as plant growth promoting rhizobacteria (PGPR) (Gopalakrishnan *et al.*, 2015). These endophytic bacteria can promote plant growth directly or indirectly. For direct growth promoting, bacteria can promote growth via nitrogen fixation, phosphate solubilization, iron chelation, and phytochrome production. For indirect plant growth promotion, bacteria will act as biocontrol agent where it suppresses pathogenic organism, induce host plant resistance against pathogenic organism and abiotic stress.

2.4 Plant Growth Promoting Activity

2.4.1 Indole-3-Acetic Acid (IAA) production

Indole-3-acetic acid (IAA) is natural auxin that accelerates plant growth and improved root and shoots development. Approximately 80% of bacteria isolated from rhizosphere are able to produce IAA (Lwin *et al.*, 2012; Gopalakrishnan *et al.*, 2015). IAA can be produced from trp through the indole-3-pyruvate pathway (IPA) found in microbes (Zhao, 2010).

2.4.2 Ammonia production

Nitrogen is one of the essential elements required for synthesis of nucleic acid, thus, it is a vital element for plant growth. Although the amounts of nitrogen are abundance in our atmosphere, plants are not able to directly assimilate it. Currently, nitrogen fertilizer is used to enhance agriculture productivity. Free-living endophytes have lower efficiency in fixing nitrogen compare to of those in leguminous plant root nodules (Hardoim *et al.*, 2015)

2.4.3 Phosphate solubilization

Phosphorus is the second most important nutrient for the plant after nitrogen. It is needed in adequate amount for essential optimal yield as it allows plants to store and transfer energy, promote roots, flower, and fruit development and allow early maturity (Duangpaeng, *et al.*, 2013). Phosphorus exists in the soil as mineral salts or incorporated into organic compound. There are reports that indicate endophytic bacteria are able to solubilize insoluble inorganic phosphate compound (Oteino *et al.*, 2015). Oteino *et al.*, (2015) also mention that research by Jilani *et al.*, (2007) and Yazdani *et al.*, (2009),

reported Phosphate Solubilizing Microbes (PBM) has the potential to reduce the rate of phosphate fertilizer application by 50% without significantly reducing crop yield.

2.4.4 Siderophore formation

Iron is an essential microelement for plant growth and is presence in soils ranging from 0.2 to 55% (20,000 – 550,000 mg/kg) and can occur in divalent (Fe^{2+}) or trivalent (Fe^{3+}), determined by pH and redox potential (Eh) (Gopalakrishnan *et al.*, 2015). Siderophore is known with high iron (Fe^{3+}) affinity thus making iron available for the plant. Fe^{3+} ions are released into the cell by gram positive and negative rhizobacteria. This reaction of reduction (iron chelation) resulted in the destruction or recycling of siderophore (Rajkumar *et al.*, 2010). In soil, microorganism siderophore production activity plays a role in improving plant development (Beneduzi *et al.*, 2012).

3.0 MATERIALS AND METHODS

3.1 Sample preparation

The root sample collected undergoes surface sterilization to remove adhering debris and microorganism attached to the root surface. Surface sterilization is needed as it ensures the bacteria isolated are endophytic bacteria. The roots were washed under running tap water for 10 minutes with soft brushing to remove soil and debris. It is rinsed again with sterile distilled water. Sterilizing agents used are 75% ethanol and sodium hypochlorite. Overall washing procedures were modified from Hallman *et al.*, (2006) sterilization procedure is summarized as follows (Table 1):

Table 1: Summary of sterilization procedure

Sterilizing agent	Incubation time
Wash under running tap water	10 minutes
70% Ethanol	5 minutes
10% Sodium hypochlorite	3 minutes
70% Ethanol	5 minutes

Between each of the steps, the root samples were rinsed with sterile distilled water and air dry under laminar for few minutes. Additionally, the root sample was passed through the flame of Bunsen burner. Sterility check was done by doing duplicates of root imprint on Nutrient Agar (NA) and aliquots of last rinsed water were spread onto nutrient agar.

The root samples were crushed (Gaiero *et al*, 2013) with additions of 1mL of distilled water and serially diluted to 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5}. Bacteria were later enumerated in Burk's agar in triplicated via spread plate method.

3.2 Preliminary screening

From the spread plate, a number of 10 isolates per plates were randomly selected and were streak onto nutrient agar to obtain pure culture for further screening. After several times of multiple streaking, 10 isolates of bacteria with distinct feature were selected for further screening based on preliminary testing which includes observation of morphological feature, gram staining, and biochemical test. The selected bacterial isolates were screened for plant growth promoting activity such as siderophore production, phosphate solubilization, indole-3-acetic acid production and extracellular enzyme screening.

3.3 Plant Growth Promoting Activities Screening

3.3.1 Ammonia production

Selected bacterial isolates were grown in peptone broth (10mL) and incubated for a period of 4 days at 27°C temperature. After 4 days of incubation, 1 mL of Nessler's reagent was added into peptone water and the colour changes were observed. Colour changes from yellow to brown indicated the production of ammonia (Cappuccino & Sherman, 1992).

3.3.2 Phosphate solubilization

Single colonies of selected bacterial isolates were transferred to Pikovskaya's agar by doing spot inoculation and incubated for a period of 7 days at 28 ± 2°C. Observations were made to check on the presence of halo zone around the bacterial colonies, which was considered as a positive phosphate solubilisation. The solubilisation index was evaluated according to the dissolution index and solubilization efficiency.

3.3.3 Siderophore production

Production of siderophore was assessed by the method of Schwyn and Neilands (1987) with modification. Instead of using liquid media, the composition of media was modified to agar plate assay (Louden *et al.*, 2011). The bacteria isolates were spot inoculated on CAS agar and incubated for 7 days at 28 ± 2°C. Observations were made to check the presence of orange colour halo zone.

3.3.4 Indole-3-acetic acid (IAA) production

Bacterial isolates were tested for IAA production according to the procedure as describe by Lwin, *et al.*, (2012). Selected bacterial isolates were grown in nutrient broth (10mL) amended with L-tryptophan (100mg/L) and incubated for 4 days at 27°C temperature.A volume of 1 mL of bacterial culture supernatant was collected from the incubating broth and centrifuged at 10 000 rpm for 15 minutes. Approximately 2 mL of Salkowski's reagent was added to the supernatant. Colour change was observed and the production of indole-3-acetic acid was measured using the colorimetric method.

3.4 Biochemical Test and Gram staining

Biochemical tests will be done according to Bergey's Manual of Systematic Bacteriology (Hoit, et al., 1989).

3.4.1 Simmons Citrate

Simmon citrate agar was prepared by dissolving the compound media in distilled water which will be dispensed into tubes that will be put in a slanting position. The tubes will be inoculated and incubated for 24 hours at room temperature. Colour changes of the media will be recorded.

3.4.2 Catalase Test

The inoculum from NB plates will be obtained and streaked on a piece of glass slide. A drop of hydrogen peroxide will be added to the bacterial smear. Observation will be recorded.

3.5 Extracellular enzyme production screening

3.5.1 Amylase

Amylase enzyme activity was determined by using starch agar (Appendix A). Bacteria were spot inoculated on agar and incubated at 27°C for 4 days. The plates were flooded with Gram Iodine solution for 1 minute and excess iodine was discarded. The presence of halo zone around colonies indicates the presence of amylase enzyme that hydrolyses starch.

3.5.2 Protease

Protease enzyme activity was determined by using Skim Milk Agar (Appendix A). Bacteria were spot inoculated on agar and was incubated at 27°C for 4 days. The pPresence of halo zone around colonies indicates the presence of protease enzyme that hydrolyses skim milk.

3.5.3 Cellulase

Cellulase enzyme activity was determined by using CMC Agar (Appendix A). Bacteria were spot inoculated on agar and incubated at 27°C for 4 days. Plates were then flooded with 0.1% (w/v) Congo red solution for 15 minute and 1M of NaCl was used to counter-stained. The presence of halo zone around colonies indicated the presence of cellulase enzyme that hydrolysed CMC.

3.5.4 Chitinase

Colloidal chitin agar (Appendix A) was prepared by using moist colloidal chitin as the carbon source. Bacteria were spot inoculated on colloidal chitin agar and incubated for 4 days at 27°C. Halo zone were observed for the hydrolysis of chitin for the detection and presence of chitinase enzyme.

3.6 Molecular Analysis

Endophytic bacteria with strong plant growth promoting activity were selected for further molecular analysis.

3.6.1 DNA extraction

Bacterial DNA was extracted using thermal lysis method. Approximately 2 mL of overnight culture were transferred into a 2 mL centrifuge tube and centrifuged at 10 000 rpm for 5 minutes. Supernatant was discarded and another 2mL of overnight culture was added and centrifuged. The supernatant was discarded. A volume of 500 µL of ultrapure water was added to the cell pellet and vortex to resuspend the cell in water. The solution was placed in 95°C water bath for 10 minutes before immediately cooled down on ice for 5 minutes. The solution was then centrifuged at 13000 rpm for 1 minute to remove debris. Supernatant containing DNA template was collected and used subsequently for polymerase chain reaction amplification.

3.6.2 Polymerase Chain Reaction

Primers used in this PCR reaction are universal primer for bacteria, PA (forward) and PH(reverse). The sequence of the primers is as shown in Table 2:

Table 2: PA and PH primer set used in PCR analysis

Primer	Direction	Annealing temperature	Sequence	Amplicon Size
PA	Forward	55°C	5'-AAGGAGGTGATCCAGCCGCA-3'	1500
PH	Reverse	55°C	5'-AGAGTTTGATCCTGGCTCAG-3'	1500

The PCR master mix used in the reaction consist of (per reaction): 8 µL ultrapure water, 6 µL of $MgCl_2$, 10 µL of buffer solution, 1 µL *Taq* polymerase, 1 µL of forward primer (PA), 1 µL reverse primer (PH), 3 µL of dNTP and 20 µL of DNA template from the